Vitamin A Tests on Edmonton Air Personnel

and

The Basic Lead Precipitate From Urine

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This is to certify that the undersigned have read and recommend to the Committee on Graduate Studies for acceptance, a thesis submitted by Kenneth W. McKerns, B.Sc., entitled:

I. Vitamin A Tests on Edmonton Air Personnel
II. The Basic Lead Precipitate from Urine

Professor.

Professor.

Professor.

Vitamin A Tests On Edmonton Air Personnel
and
The Basic Lead Precipitate From Urine

Kenneth Wilshire McKerns

A thesis submitted in conformity with the requirements for the degree of Master of Science by the University of Alberta.

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PART I Vitamin A Tests on Edmonton Air Personnel

PART II THE Basic Lead Precipitate from Urine

Vitamin A Tests on Edmonton Air Personnel

The objects of the present investigation are:-

1. To ascertain the extent of "night-blindness" among men in the R.C.A.F.

2. To look for those individuals having an exceptionally good night or twilight vision.

3. To study methods of correcting the condition of "night-blindness", with special reference to the use of Vitamin A oils and of carotene.

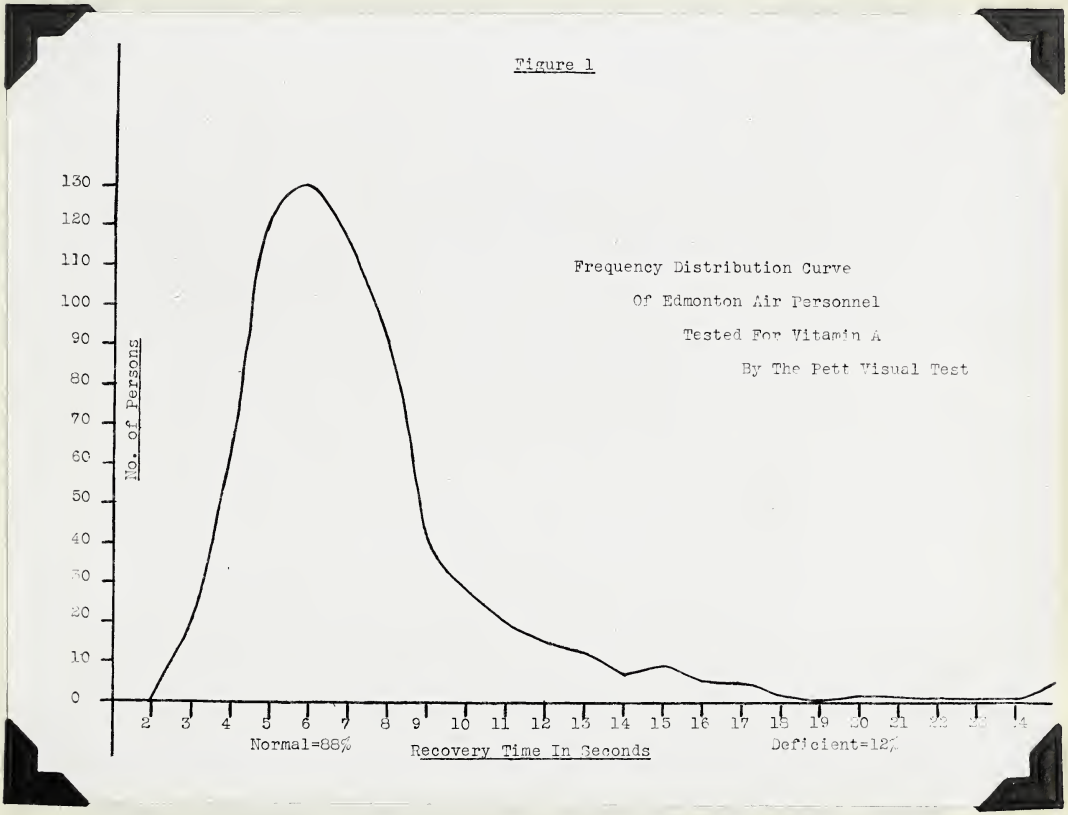
The investigation was carried out on four Edmonton elementary training schools, covering a period from the first of June to the end of September, 1941. The testing was conducted either at the training school or when more convenient at the Department's laboratories.

Basis of the Test:-

Vitamin A is a necessary factor in the normal behavior of the retina. Carried in the blood stream to the retina, Vitamin A forms a complex with a protein, the visual purple of the rods. Visual purple is destroyed by light and until regenerated the individual is relatively blind to dim lights. Unless the eye receives a continuous supply of Vitamin A the regeneration is incomplete or delayed.

Assuming a relationship between the rate of regeneration of visual purple and the amount of Vitamin A in the blood, many tests for measuring Vitamin A deficiency have been proposed. (3)

Figure 1



In this Department a test, both sufficiently accurate and rapid enough to permit the treating of large numbers of people, was developed by Dr. L. B. Pett. (1) This visual test has been correlated with blood analysis for the vitamin. (2)

Method of Testing:-

In brief, this test consists in identifying a dim bar of light after looking at a bright light for 30 seconds. The time taken to identify the bar of light, after the bright light is turned off, is noted, and this represents the "recovery time". Two or three tests are repeated at one minute intervals and the mean of the values is taken. People with recovery time of 10 seconds or less are considered normal.

The men were paraded for the test and one operator handled thirty men an hour. To those found deficient 100,000 I.U. of Vitamin A was given - either as carotene (10 capsules) or fish oils (3 capsules). Those treated men were retested later and treatment continued until normal ranges were obtained or until lowering of their values could be obtained.

Results and Discussion

The frequency distribution curve of the total number tested is shown in figure 1. The graph shows a peak of 6 seconds and 88% of the men fall in the normal range.

This may be compared with results from over 4000 persons tested at this University, showing only 43% in the normal range with a peak at 10 seconds. The men having exceptionally good dark adaptation, that is, those with recovery times of 4 seconds or less, made up approximately 12% of the total. These men would possibly make good night fighters. (4)

Table I shows recovery times of the personnel of each school tested. The results vary widely in different schools - from 3% deficient in the Radio School to 24% deficient in No. 2 A.O.S. and the deficiencies are high enough to warrant attention in men engaged in night operation.

Response to Treatment:-

Treatment is usually carried out by giving Vitamin A from some fish liver oil, or by giving the provitamin carotene. Since there is some question about the adequacy of supplies of fish liver oils in Canada and Britain, during the war, it is considered especially important to consider the results with carotene, which is prepared from vegetables.

Table 2 gives details of the response to carotene, Table 3 to Vitamin A and Table 4 some individual results on repeated treatments.

Results with Carotene:- First dosage 100,000 I.U. in 3 days.

Sixty percent of all deficients did not return to normal with the first dosage. However, a lowering of the recovery time was usually noted and the continued treatment for those still deficient ultimately restored all to normal except 6%. Of those with moderate deficiencies only 19% did not respond to the first treatment. Table 4 shows the importance of using bile salts in some cases needing treatment. Also shown are a few cases not restored to normal by carotene, even with associated riboflavin and ascorbic acid. Vitamin A was always effective.

Table 1

Dark Adaptation Test - Recovery Times In Seconds

Normal								Border-Line					Deficient										
3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	-Seconds
1	8	17	19	26	29	9	13	12	9	7	5	5	4	3			1	1	1			2	-Number
																							No.2 AOS - 50/171 = 24% Deficient
	5	9	7	11	7	7	4	3	2	2	1	1					1					1	
																							No.16 EFTS - 11/59=15% Deficient
	11	17	22	25	24	12	4	2						1	1								
																							Radio School - 4/119=3.4% Deficient
18	41	77	81	53	30	12	7	3	4	4	1	2	1	1									
																							No.4 ITS - 16/357 = 4.5% Deficient

Total Number Tested = 706
Deficients = 80 Percent Deficient = 12

Table 2

First Column - First Test Time

Second Column - Recovery time after 100,000 I.U. Carotene

Response to Carotene:

	Seconds		Additional Dosage	Recovery Time
	From	To		
R92922 - Allen	17	15	100,000 I.U.	7
404649 -Bowman	11	7.5		
404603 -Daniels	13	6		
404624 -Given	12	7		
403108 -Henry	13	8		
403417 -Kennedy	12	5		
406251 -Lawton	11	8		
403478 -Lurie	17	10		
R86841 -Morgan	11	15	100,000 I.U.	4
404869 -Mustoe	17	5		
403397 -O'Tiodan	14	14	100,000 I.U.	8
401246 -Richardson	14	14	100,000 I.U.	8
-Richardson	12	5		
402754 -Ridley	11	4		
403469 -Unger	11	9		
R92581 -Waring	15	12	100,000 I.U.	7
-Wilson	15	12	100,000 I.U.	7
401343 -Wood	16	5		
400610 -Wilson	11	3.5		
401228 -Allen	17	15	100,000 I.U.	7
R91973 -Hicks	11	9		
R92544 -McFadden	15	8		
R95414 -MacGarva	12	9		
R87314 -Pozer	11	10		
R87370 -Thompson	11	10		
R90313 -Clement	20	10		
R90529 -Kidd	13	9		
R86811 -McCartney	13	9		
R88848 -Payton	14	5		

Table 2 (cont'd)

Response to Carotene - 100,000 I.U. in 3 days

R110288 - Payne	15	5			
R110218 - Haughey	13	8			
R105891 - Ross	12	7			
R103761 - Salmon	17	12			
R107768 - Waters	14	10			
R107693 - Coffey	13	9			
R102095 - Maxwell	30	16			
R110919 - Smith	11	6			
R110998 - Wilson	13	8			
R98961 - Milne	12	7			
R106441 - Kane	16	9			
R106031 - Orloff	12	8			
R95410 - Robb	15	6			
R106072 - Ruvinsky	13	7			
R105806 - Wallace	14	18	100,000 I.U.	11	
" "			100,000 I.U.	12	
" "			100,000 I.U.	12	
R114702 - Kirkhan	11	8			

Response to Carotene - 60,000 I.U. in 3 days

R 92862 - Michin	18	5	
R100672 - Tokeruk	11	5	
- Allen	11	9	
R103620 - Whenham	19	15	After repeated large dosages no return to normal level.

Table 3

Response to Vitamin A - 100,000 I.U. in 3 days

	From	To		
403838 - Cummins	13	7		
401349 - Lowe	12	9		
401594 - Wilmouth	14	7		
405150 - English	14	10		
405442 - Gibson	12	9		
403132 - Patterson	14	10		
405443 - Gray	17	5.5		
411004 - Cleland	15	15	200,000 I.U. + bile	-5
405457 - Stuart	18	12	" " " " " "	-5
403146 - MacKenzie	16	12	" " " " " "	-10
403470 - Henry	25	13	" " " " " "	-9
404931 - Boyle	15	15	" " " " " "	-5
403312 - Braithwaite	25	16	" " " " " "	-8

Response to Vitamin A - 80,000 I.U. in 3 days

1263189 - Austin	12	6		
R92159 - Abbott	12	9		
R77126 - Dobson	12	8		
R60563 - Frankish	13	8		
R77270 - Gillman	12	9		
R71482 - Lamont	11	8		
R65430 - Powell	13	4		
R86896 - Rigden	11	4		
R92182 - Robinson	13	16	+ 1 capsule	-7
R69209 - Simpson	20	9		
R85479 - Southae	11	8		
R86833 - Willis	14	9		

Table 4

First Dose: - Carotene 100.000 I.U. in 3 days

McKennon - 12 - 14 + 100,000 I.U. carotene in 2 days - 14 + Vit. A 100,000 in one day - 10

Powell - 11 13 + 100,000 I.U. carotene in 2 days - 14 + carotene 100,000 - 15 + Vit. A 100.000 - 12 + Vit. A 100,000 + bile in 2 days - 9

Stoval - 26 - 30 + 100,000 carotene - 25 + 100.000 Vit. A in one day - 20

Landy - 20 - 25 + carotene 100.000 + riboflavin 10.mg. + Vit. C 250 mg. all in 3 days - 13 + carotene 100.000 - 24 + Vit. A 100,000 in one day - 16 + 100,000 A in 3 days - 15 + Vit. A + bile in 2 days - 18

Levett - 22 - 22 + carotene 100,000 + Vit. C 250 mg. + riboflavin 10 mg. all in 3 days - 17 + carotene 100.000 - 22 + Vit. A in one day - 14 + Vit. A 100.000 - 14 + Vit. A 100.000 + bile in 2 days -20

Fairway - 11 -11 + 100,000 carotene in 3 days - 14 + Vit. A 100,000 - 11 + Vit. A 100.000 - 10

Gummerson - 21 - 15 + 100.000 carotene in 3 days - 14 + Vit. A 100.000 in one day - 9

Brown - 11 - 15 + carotene 100,000 in 3 days - 11 + Vit. A 100,000 in one day - 8

Picker - 18 - 11 + carotene - 19 + carotene 100,000 in 3 days - 12

Results with Vitamin A:- 100.000 I.U. in 3 days

Nearly 40% of all deficients did not return to normal with the first treatment. Of cases with recovery times from 11 to 15 seconds, only 19% did not return to normal with the first 100.000 I.U. given, this being the same as with carotene.

Only five deficients (6%) in all the numbers treated showed no lowering of their recovery times with repeated large dosages.

No one dosage is satisfactory to cure all cases. Frequent retesting of all deficients with additional dosages when required is advised.

Summary

1. Using the Pett Vitometer it was possible for one operator to test air personnel at the rate of 30 men an hour.

2. Of the total number of men (706) tested, 12% were found to be deficient in Vitamin A.

3. The percentages of deficients varied from 3 to 24% in the various schools or groups tested.

4. Twelve percent showed exceptionally good dark adaptation.

5. With 100,000 I.U. of carotene over a three-day period 40% of the deficients were brought to normal. Further treatment restored most of the remainder.

6. With 100,000 I.U. of Vitamin A given as fish liver oils over a three day period, 60% of all deficients treated were brought to a normal recovery time.

7. Ninety-four percent of all deficients were brought to a normal level after one or more treatments. No one dosage was found to be effective in all cases. Bile salts were sometimes necessary. Retesting was the only means of being certain of the results.

Conclusions

While the number of persons lacking Vitamin A among the air personnel is low, there are still enough to be an important factor. The testing period included the months where the lowest number of deficiencies have been found in other investigations. Winter, with its lack of fresh vegetables and fruits shows a marked rise in the numbers of deficients found. Since the number requiring treatment is small, indiscriminate feeding of Vitamin A to all personnel would be wasteful. This would be especially serious in view of the uncertainty of supplies of fish oils.

Acknowledgements

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Carotene capsules used in treatment were supplied by John Wyeth and Brother (Canada) Limited: and Vitamin A capsules by the Upjohn Company.

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THE BASIC LEAD PRECIPITATE FROM URINE

Introduction

A number of substances of doubtful composition, possibly peptides or partially oxidized protein fragments, have been isolated from human urine. These substances, escaping complete metabolism by the body, are termed oxyproteic, antoxyproteic, and alloxyproteic acid by their isolators, Bondzynski, Dombrowski and Panck (1). In the same class is the uroferric acid of Thiele (2).

Subsequent workers are not in agreement with regard to the nature or the hydrolytic products of the so-called proteic acids. Even from the writings of the original authors, no hint is given that these substances were isolated, other than in an amorphous, impure state.

However, according to Folin quoted by Allen (3), three to seven percent of the total urinary nitrogen is still unaccounted for, other than as proteic acids. A similar small part of the urinary sulphur is also attributed to these acids.

The elementary analyses of the acids as given by the authors (3) along with their reactions to Erlich's diazo test, are:

	C	H	N	O	S	diazo
Oxyproteic acid	39.62	5.64	18.08	35.54	1.12	-
Antoxyproteic acid	43.21	4.91	24.40	26.33	0.61	+
Alloxyproteic acid	41.33	5.70	13.55	37.28	2.19	-
Uroferric acid					3.46	

They do not give the common protein tests.

Since there is much uncertainty about the composition of these substances further research into the nature of the acids is clearly called for. The author's isolation procedures were found unworkable and no serious attempt to follow their laborious and detailed instruction was made.

The purpose of the present investigation is to elucidate the nature of the substances in the basic lead acetate precipitate of normal urine. It is in this fraction that the substance responsible for Ehrlich's diazo test in the urine of fibrile patients is found: a substance which has been called urochromogen by Weiss (4), as it appears to be the chromogen of the normal urinary pigment, urochrome. Various unsuccessful attempts have been made in this laboratory and elsewhere to isolate the substance in question. It is relatively unstable and observations have suggested that its instability is increased with high H-ion concentration. Under the usual conditions of obtaining the basic lead precipitate, when the lead salt is freed with hydrogen sulphide the resulting solution, is always very strongly acid. It had previously been observed that when sulphuric acid is used to free the lead salt (instead of hydrogen sulphide), the lead is not all freed when the solution is just acid to Congo Red. It was thought that this acid effect might be due to the so-called

proteic acid, to which group it is possible, urochromogen belongs. But as the freeing of the lead salts by hydrogen sulphide or sulphuric acid was inevitably accompanied by a high H-ion concentration, it was thought possible to exchange the lead in the salt for potassium by shaking with a potassium sulphate solution. On such an assumption, which has subsequently proved to be erroneous, and by certain fractionation procedures, a crystalline potassium salt was prepared from this fraction. The nature and origin of this potassium salt is described.

So far as present observations have gone it also appears that the above mentioned "acid effect" is due largely to hydrochloric acid arising from chloride in urine: as much of the chloride may not be precipitated in the acid lead fraction of urine. Such observations have altered our approach to the problem. Urine must be freed from chloride before proceeding with the lead fractionation.

EXPERIMENTAL

Lead Fractionation of Urine

In the initial stages of the investigation, the method of collecting the urine was the same as one used in this laboratory when collecting urine from typhoid patients, for determining "urochromogen " levels. The quantity of lead

acetate used ensured an excess for urines of all concentrations.

Urine was collected with one third of its volume of 40 percent lead acetate. After standing overnight, the supernatant was poured off and the precipitate discarded. Two normal potassium hydroxide was added to the clear greenish supernatant, to bring the pH to 7.5. The lead precipitate, insoluble at this pH, was allowed to settle overnight in the refrigerator. The supernatant was siphoned off and discarded and the precipitate collected in centrifuge bottles, washed twice with water and three times with acetone to obtain a dry lead precipitate. (by this means it is possible to store the lead salt of urochromogen over long periods without decomposition.)

Five grams of the lead salt was ground with 15 ml. of absolute methanol and 7.5 ml. of 5N sulphuric acid in absolute methanol. This shifted the pH to slight acidity with Congo Red. A saturated solution of potassium sulphate was added until there was a slight excess of sulphate ion. After centrifuging off the lead sulphate and evaporating in vacuo, to the beginning of crystals, the solution was dried in a desiccator. The dark brown mass was extracted with dry, alcohol-free ethyl ether: the extract was poured into a small basin and allowed to volatilize in the open. This gave a yellow, slow-drying sticky mass, showing a fern-like crystal pattern.

The residue from the ether extraction was ground with absolute ethanol, washed several times with absolute ethanol, evaporated under reduced pressure, and dried in a vacuum desiccator.

The residue was then extracted with absolute methanol. The methanol soluble fraction was evaporated under reduced pressure to the beginning of crystallization, some absolute ethanol added and left to crystallize in the refrigerator. This gave well formed crystals, only slightly colored.

Various attempts to purify the crystals from the methanol extraction were tried, including recrystallization from hot methanol. Finally this portion was dried in the desiccator, dissolved in methanol, and heated to near boiling with animal charcoal. This gave an almost colorless liquid which on cooling gave white crystals. A melting point determination showed charring at 170° C and fusing at 220° C.

The preparation was modified as follows: after the solution was freed of excess lead ion and diluted with water it was boiled with bone charcoal, centrifuged and boiled again with charcoal. The water clear solution was evaporated under reduced pressure, dried in a desiccator, and extracted with absolute ethanol. The residue was extracted with absolute methanol and the remaining methanol insoluble portion dissolved in hot water. The methanol

soluble portion after drying in a desiccator was re-crystallized from hot methanol, vacuum filtered, and washed with absolute ethanol. This gave white clear plate-like crystals, melting point 228°C with no charring preceding melting. The crystals were very soluble in hot or cold water, very soluble in hot absolute methanol, insoluble in ether or benzene. After fusion with sodium, sulphur was strongly positive, nitrogen and phosphorus and halogens were negative.

Crystalline Potassium Salt

On ignition in a platinum boat the substance melted, and without appreciable charring left a large white residue in which only sulphur and potassium could be detected. Evidently the crystals isolated were the potassium salt of a relatively simple substance, probably a sulphonic acid. The ease with which the potassium salt hydrolized to sulphuric acid suggested the presence of ethereal sulphates. To demonstrate this, to a solution of the potassium salt in water was added one third of its volume of strong hydrochloric acid. The solution was boiled for five minutes and barium chloride added--a white cloud of barium sulphate formed. The compound was apparently a potassium salt of a sulphonic acid of the type RSO_3K.

Attempts were now made to obtain the free acid from this potassium salt. To an aqueous solution of the salt

lead acetate solution was added and the pH raised to about 8.0. There was no precipitate.

To some of the lead salts suspended in water 2N sulphuric acid was added and the excess sulphate was removed with barium hydroxide. After centrifuging off the barium sulphate and drying the free acid in a desiccator nothing was obtained.

Hydrogen sulphide was passed into a suspension of the lead salt in water and the excess hydrogen sulphide removed by boiling. This solution was acid to litmus and Congo Red paper and free sulphuric acid was present since it gave a positive test with barium chloride and hydrochloric acid.

The only conclusion possible from the above findings, and assuming no significant decomposition of the substances in question, is that this potassium salt was introduced from our reagents.

Further examination of the potassium salt proved the absence of nitrogen by micro-Kjeldahl determination.

By the method of Warunis (5) sulphur determination showed 21.77 and 21.07%.

Several methods were tried for the determination of potassium. The method of Pregl (6) failed in our hands to give uniform values. A dipicrylamine method (7) also failed to give consistent values.

Finally the chloroplatinate method (8) was adopted and yielded values of 25.96, 26.17, 25.90 and 25.93% K. Through the kindness of Mr. J.M.Lockwood, Division of Chemistry, National Research Council, Hydrogen and Carbon values were made available.

The salt in question is thus CH_3SO_3K, as proved by the following average analysis:

	C	H	S	K
Found	8.52	2.26	21.42	25.99
Theory	7.99	2.01	21.34	26.57

This material was formed in the mixed reagent used, composed of concentrated sulphuric acid and methanol, as proved by isolation.

Conditions for Lead Fractionation

a. Lead Requirement

Having disposed of this misleading finding, a return was now made to the lead fractionation with a view first to defining conditions. The first of these was to determine the requisite amount of lead required by the urine. The principle used here is as follows: Ammonium hydroxide is chosen as base to raise the pH of aliquot portions, as there is little evidence that any lead salts are dissolved by addition of excess ammonium hydroxide, whereas, it is well known that there is appreciable resolution of lead salts in excess sodium hydroxide or potassium hydroxide. Accordingly, 2 ml. aliquot portions of

the urine to be fractionated were set up and varying amounts of 40% lead acetate added to each. A definite excess of ammonium hydroxide was then added to each. The mixed tubes were centrifuged and to a few drops of the supernatant from each, 5 N sulphuric acid was added. A precipitate of lead sulphate then shows an excess of lead, and the amount of lead acetate to be added to the main urine can thus be determined precisely.

The requisite amount of 40% lead acetate was added to the urine and the pH adjusted to near 5.0. The precipitate formed here is composed largely of lead salts of phosphate, sulphate, chloride, carbonate, and various organic materials, such as uric acid and urinary pigments. This precipitate is separated and discarded.

To the filtrate, 2 N potassium hydroxide is added to raise the pH to about 9.0. This precipitate is separated and washed, suspended in water, and treated with hydrogen sulphide. The lead sulphide is removed, and the hydrogen sulphide removed from the filtrate.

Interference of Chloride

The filtrate at this point is invariably found to be very strongly acid, due to the presence of hydrochloric acid.

We had erroneously assumed (on the basis of solubilities of lead chloride in water) that there would be no appreciable lead chloride in a well washed basic lead

precipitate. This error was corrected by the isolation of potassium chloride crystals from this fraction.

We have found that in urines where the concentration of sodium chloride is less than 4 gm/l, that chloride is precipitated in all pH ranges. Dilution of urine well below the solubilities of lead chloride in water is no assurance that the lead precipitates will not contain chloride.

The presence of lead chloride in the basic lead precipitate appreciably increases the bulk of the precipitates and leads to confusion in reaction as previously mentioned. This has no doubt been a confusing factor in much of the earlier work on proteic acids. It was thus decided that chloride should be removed from all urines before undertaking the lead fractionation. This can readily be done as follows:

Removal of Chloride from Urine

1 ml. urine delivered into a 50 ml. centrifuge tube is diluted with approximately an equal volume of water, 2 ml. of the standard silver nitrate-nitric acid mixture added (1 ml.=0.01 gm. NaCl), mixed, and 1 drop of caprylic alcohol to depress the solubility of the AgCl is added. (9) The tube is centrifuged and titrated with standard thiocyanate and the silver nitrate requirement thus determined.

To the urine to be fractionated, and with the pH definitely on the acid side of neutrality, an aqueous solution of silver nitrate (1 ml.=0.1 gm NaCl) is added according to the above determination. If excess is avoided the precipitate readily coagulates and can be easily separated.

A further advantage in thus removing chloride lies in the fact that the lead requirement can be more accurately determined by the method already described.

The lead precipitate thus obtained in the range pH 5.0 - 9.0 yields a solution after removal of lead and hydrogen sulphide having a pH 3 - 4.

Fractionation on Chloride Free Urine

This solution has been refractionated by Pb in the pH ranges 5.0 - 6.8 and 6.8 - 9.0. The precipitate obtained in the more acid range is small and highly pigmented. The main material under investigation is thus to be found in the pH range 6.8 - 9.0.

This fraction yielded no crystalline material on evaporation and so far it has not been possible to prepare a crystalline salt. That the fraction is still complex is indicated by its behaviour in the silver-baryta fractionation to be described. A mercury precipitation was carried out as follows:

Mercury Precipitation

Mixed urines in approximately 2 litre lots were put through the lead fractionation procedure and the aqueous solution of free acid thus obtained was subjected to a mercury fractionation. The mercury requirement of each urine was determined on an aliquot portion: to 1 ml. of the free acid solution was added 5% mercuric acetate until no further precipitate was obtained. The mixture was adjusted to just acid with 5% sodium carbonate solution. Another drop of mercury was added and if there was no further precipitate sufficient mercury had been added. A red precipitate of mercuric oxide indicated an excess of mercury. The main precipitate was washed twice with water containing a little sodium carbonate and the supernatant and washings saved. The precipitate suspended in water was freed with hydrogen sulphide and the mercuric sulphide centrifuged off and discarded after one washing. The filtrate and washing was aerated with a stream of carbon dioxide and concentrated in vacuo to dryness. This gave a yellow colored mass showing some fern-like pattern. The material was extremely soluble in water, insoluble in absolute alcohol, but soluble in 95% alcohol.

One sample of urine put through the preceding two fractionations showed the following:

	Pb ppte. 6.8-9.2 freed $\bar{c}$ H_2S	Hg ppte. freed $\bar{c}$ H_2S	Supernatant from Hg Ppte.
Total S	34.8 mg.	30.0 mg.	0.00

The total sulphur analysis shows that most of the sulphur precipitated by lead at pH 6.8-9.0 is reprecipitated by mercury.

Adding absolute alcohol slowly with warming to the concentrated solution from the mercury fractionation procedure gave a sticky mass of yellow crystals. Attempts to remove the sticky yellow pigments were unsuccessful since the material was unstable and oxidized to a brown mass.

SILVER FRACTIONATION

The freed mercury precipitates from fresh urines samples were put through a silver fractionation. 10% silver nitrate was added to the solution from a mercury fractionation until an excess was present as shown by the formation of an immediate brown spot test with concentrated barium hydroxide solution. The light precipitate that formed was left in solution. A hot saturated solution of barium hydroxide was added until the solution had reached a pH of 7.0. The relatively small precipitate was centrifuged off and washed. Excess barium hydroxide was then added to the filtrate and the precipitate similarly collected. The two precipitates and the filtrate were

made acid to Congo Red with sulphuric acid and the silver freed with hydrogen sulphide. The hydrogen sulphide was removed with a stream of carbon dioxide and by evacuation on the vacuum pump. Excess sulphuric acid was removed from the three solutions with barium hydroxide solution. The solutions were reprecipitated with lead acetate and the lead salts after washing were freed in the usual way. The three solutions were evaporated to dryness on the vacuum pump.

Silver precipitation revealed the mixture to be complex. The freed material from the first silver precipitation yielded a small quantity of heavy brown liquid. The second freed precipitate was larger and gave a yellow amorphous powder. About half of the bulk of the mixture was obtained from the filtrate as a red brown syrup. No crystals were obtained from the above material.

Sulphur Analysis

(a) Methods

Inorganic ethereal and neutral sulphur and total sulphur was determined on the native urine and on the various lead fractions. The results were expressed as sulphur.

The Fiske's Benzidine titration method (10) was tried first and found satisfactory only for native urine. Even on native urine Benedict's total sulphur reagent of copper nitrate and potassium chlorate, is of doubtful value as an oxidizing agent. On applying the benzidine titration

method to the filtrates after the removal of each lead fraction, acetate, nitrate and phosphate ions titrated with NaOH in the same way as benzidine sulphate.

Gravimetric methods were adopted. Sulphur determinations were done on the native urine and on the lead precipitates collected at pH 5.0 and pH 9.0 and on the supernatant. Each precipitate was collected in a centrifuge bottle, washed once with water and the washing added to the filtrate. The precipitates and the filtrate were freed of lead with hydrogen sulphide and the hydrogen sulphide removed with carbon dioxide.

The Warunis (5) method for total sulphur was found to be very accurate when total sulphur was determined in pure cystine. However, applied to urine, the method was not satisfactory: urine adhered to the crucible during evaporation and complete fusion was questionable. The results on duplicate samples varied as much as 4%.

Denis' modification of Benedict's method (11) using a solution of copper nitrate, sodium chloride and ammonium nitrate, did away with spattering and duplicate results checked closely.

Inorganic and ethereal sulphur was determined by Folin's method (12). To 25 ml. of urine and 15 ml. of water are added 6 ml. of 3N HCl and 10 ml. of 10% barium chloride. After standing for about an hour the

solution is filtered through a weighed Gooch crucible and the inorganic sulphur determined. The filtrate is boiled gently for thirty minutes, cooled, and filtered and the ethereal sulphur determined. The organic sulphur was arrived at by subtracting the sum of the inorganic and ethereal from the total sulphur.

Every series of determinations showed a loss of sulphur. The total sulphur of the lead fraction at pH 5.0 and 9.0 plus the supernatant sulphur was less than the total sulphur of the native urine.

When acidified urine was boiled hydrogen sulphide was generated, as proved by blackening a solution of lead acetate. Also urine evaporated to dryness with the oxidation mixture gave rise to some hydrogen sulphide.

Four total sulphur determinations were done on the same urine sample:

I (a) 25 ml. native urine---23.1 mg. S
(b) " " " " ---23.0 " S

II (a) 25 ml. urine boiled with HCl---20.1 mg. S
(b) " " " " " " ---21.2 " S

(b) Quantitative Results:

Total sulphur by the Denis modification.

Inorganic and ethereal by Folin's method.

Results are expressed as the average of two determinations.

175 ml. urine (night specimen K.M.) sp. gr. 1.017

Total Sulphur

	Mg. S	
Native urine	79.50	
pH below 5.0	54.25	74.50
pH 5.0 to 9.0	6.50	
Supernatant	13.75	

Sum of Inorganic and Ethereal:

	Mg. S	
Native urine	67.70	
pH below 5.0	53.50	60.15
pH 5.0 to 9.0	3.13	
Supernatant	3.52	

Neutral Sulphur

	Mg. S	
Native urine	11.80	
pH below 5.0	0.75	14.86
pH 5.0 to 9.0	3.37	
Supernatant	10.74	

Urine Sample (W.E.) 175 ml. sp. gr. 1.026
(Average of three determinations)

Total Sulphur

	Mg. S	
Native urine	170.0	
pH below 5.0	111.0	138.0
pH 5.0 to 9.0	7.0	
Supernatant	20.0	

Inorganic and Ethereal

	Mg. S	
Native urine	128.0	
pH below 5.0	109.5	121.0
pH 5.0 to 9.0	3.4	
Supernatant	8.1	

Neutral Sulphur

Native urine	42.0	
pH below 5.0	1.5	17.05
pH 5.0 to 9.0	3.7	
Supernatant	11.90	

It is thought the loss of sulphur in the above determination occurs in the inorganic sulphur fractions below pH 5.0, probably due to the incomplete freeing of the very stable lead sulphate. However, it is interesting to study the neutral sulphur partition. In each determination approximately three quarters of the neutral sulphur was not precipitated by lead. Presumably the one quarter of the neutral sulphur precipitated by lead at pH 5.0 to 9.0 corresponds to the alloxyproteic acid fraction.

(c) Loss of Sulphur

Even though organic sulphur is lost as hydrogen sulphide when boiling with HCl for the determination of ethereal sulphur this loss is irrelevant since the

organic sulphur is arrived at by the difference between the inorganic and the total sulphurs. To account for the loss of sulphur the following possibilities were considered:

1. The method of Denis may be inapplicable:
   a. To determining the S-S linkage
   b. To determining the S-H linkage.
2. Precipitation with lead acetate and subsequent freeing with hydrogen sulphide may bring about a loss.
3. A loss through the incomplete freeing of the lead salt, especially the lead sulphate.

1. Denis' method was used on the S-S linkage

500 mg. of cystine were dissolved in 100 ml. of water (acidified with HCl to dissolve the cystine). Total sulphur determinations were done on 15 ml. portions.

Calculated result = 19.97 mg. S

As determined:

19.96
20.20
19.08
20.82

Average 20.02 mg. S

Denis' method applied to S-H linkage

526 mg. of cysteine HCl dissolved in 50 ml. of water. 10 ml. portions used for analysis.

Calculated result = 21.5 mg. S

As determined:

22.2
22.6 Average 22.4 mg. S

2. 526 mg. of cysteine HCl were dissolved in 50 ml. water. To 6 ml. of the solution were added 0.5 ml. of 40% lead acetate, and hydrogen sulphide was bubbled through. The lead sulphide was washed twice with water.
Calculated result = 12.80 mg. S
As determined:

12.95
13.10 Average 13.05 mg. S

3. To 150 ml. urine (W.E. sp. gr. 1.025) was added 30 ml. of 40% lead acetate solution. The precipitate was washed and the washing added to the supernatant. The supernatant and the precipitate were freed of lead with hydrogen sulphide. In each case the lead sulphide was washed and treated again with hydrogen sulphide after thoroughly dispersing the sulphide in water. Total sulphur determinations were done on each fraction.

Results

Native total S - 131, 129 Average 130 mg. S

Precipitate - 66.9, 64.3 Average 65.6 mg. S

Supernatant - 34.5, 33.9 Average 34.2 mg. S

Loss = 130 - 99.8 = 30.2 mg. or 24.5%

Urine sample (W.E. 120 ml. sp. gr. 1.023)

Native - 102.0, 101.7 Average 101.8 mg. S

Precipitate - 21.9, 21.3 Average 21.6 mg. S

Supernatant - 60.9, 60.2 Average 60.6 mg. S

Loss = 101.8 - 82.2 = 19.6 mg. or 19%

Thus it would appear a loss of sulphur occurs because of an inability to completely free the lead salt with hydrogen sulphide. Either the pH is not low enough to free the lead sulphate or the physical properties of the urinary lead salts interfere with the complete freeing.

Effect of pH on Freeing Lead Sulphate

10 ml. portions of a solution containing 45.7 mg. of potassium sulphate were taken for analysis. Lead acetate was added to each and hydrogen sulphide was bubbled through to free the lead.

Sample 1--pH less 1.0	Sample 2--pH 7.0
10 ml. sulphate solution	10 ml. sulphate solution
10 ml. dil. HCl (1 ml. conc. HCl/100 ml. water)	10 ml. water
1 ml. 40% lead acetate	1 ml. 40% lead acetate

Sample 3--pH greater 9.0

10 ml. sulphate solution

10 ml. water

0.35 ml. 2N KOH

1 ml. lead acetate

A calculated amount of 2N KOH sufficient to neutralize the sulphuric acid freed from the lead sulphate was added to Sample 3, before hydrogen sulphide was passed. The initial pH of this solution was far higher than any encountered in the similar treatment of urine fractions. The three samples were

centrifuged and the lead sulphides washed twice with water and the washings added to the supernatants. Hydrogen sulphide was removed with a stream of carbon dioxide and by evacuation on the vacuum pump.

The supernatant from sample three had a final pH of five. Duplicate sulphur determinations were done on the three supernatants.

Results

Calculated	=	8.41 mg. S
Average found	1.	8.42 mg. S
	2.	8.43 mg. S
	3.	8.41 mg. S

Thus some physical property of the urinary lead salts interferes with the complete freeing of lead sulphate.

Ergothioneine-Like Substances

Sullivan and Hess (13) claim to have isolated ergothioneine-like material from normal urine. This material behaved like ergothioneine in that it reacted with the Folin-Marenzi (14) uric acid reagent and gave a red color with Hunter's diazo reagent (15). Analyzed for nitrogen and sulphur it gave nitrogen 15.26%, sulphur 11.95%, closely agreeing with the calculated values for ergothioneine hydrochloride: nitrogen 15.81%, sulphur 12.07%. The free base melted at 261° and decomposed between 285-290°. The authors stated at the end of their paper that most of the material isolated did not seem to be ergothioneine. If the claims of the authors could be substantiated some light might be thrown on the nature of the unknown sulphur compounds in urine.

Isolation Procedure

A modified Hunter and Eagles (16) procedure, used for the isolation of ergothioneine in blood, was followed. Four litres of normal urine was brought to pH 5.0 by the addition of glacial acetic acid. Goulard's reagent (17) was added until a test portion of the

supernatant showed an excess of lead on testing with sulphuric acid. The filtrate was freed of lead by hydrogen sulphide and the hydrogen sulphide removed with a stream of carbon dioxide. A saturated alcoholic solution of mercuric chloride was added to the filtrate until no further precipitation occurred. The mercury precipitate was washed twice and freed with hydrogen sulphide. The mercury sulphide was thoroughly washed and the washings added to the filtrate. The filtrate was freed of hydrogen sulphide as before, made to 0.5N by the addition of 10N sulphuric acid and precipitated with 20% phosphotungstic acid in 0.5N sulphuric acid until precipitation ceased. The pinkish precipitate was allowed to stand in the refrigerator over night. The phosphotungstic precipitate was centrifuged off in the morning, keeping the temperature as low as possible and the precipitate was washed twice with ice cold 0.5N sulphuric acid to which had been added a little of the phosphotungstic acid solution (5 mls./ 100 ml. of 0.5N sulphuric acid).

The precipitate was freed with a hot saturated solution of barium hydroxide, the latter added until the solution tested definitely alkaline to litmus. The filtrate was carefully freed from barium by adding 5N sulphuric acid until the end of precipitation and then a quantitative removal of barium and sulphate was accomplished with 0.1N solutions of barium hydroxide and sulphuric acid. The filtrate was concentrated under reduced pressure to about 15 ml. and a great deal of material separated out. The great bulk of this material was creatinine. However it gave a positive diazo test for histidine and a small amount of histidine was isolated from the material. The material freed from the mass of creatinine with picric acid gave a stable dark red nitroprusside test, instead of the characteristically unstable purple red reaction with sulphydryl groups.

Benedict, Newton, and Behre (18) showed that ergothioneine is precipitated by 5% silver lactate in 5% lactic acid but is not freed by washing with a 10% solution of sodium chloride in 0.1N hydrochloric acid. Ergothioneine can thus be separated from uric acid which is extracted by acid sodium chloride from the silver precipitate.

The concentrated filtrate from the freed phosphotungstic acid precipitate and with the separated creatinine removed was treated with silver lactate in slight excess. The silver precipitate was centrifuged off and the supernatant saved. The silver precipitate was washed several times with the acid sodium chloride solution until a supernatant gave a negative reaction with the Folin-Marenzi uric acid reagent. The precipitate was then boiled for five minutes with 10 ml. of 0.5N hydrochloric acid and the filtrate left over night in the refrigerator--nothing separated out. The filtrate was concentrated to approximately 1 ml. and an equal volume of absolute alcohol was added slowly to the warmed solution. After standing for several hours in the refrigerator the small amount of white material that had separated out was collected in a small sintered glass funnel, washed with absolute alcohol, and dried in a desiccator. This material proved to be mostly uric acid and showed no traces of either ergothioneine or histidine. Evidently the silver lactate precipitate was not washed enough with the acid sodium chloride solution to remove all the uric acid.

It was also found the silver lactate reagent would precipitate part of the histidine from a fairly concentrated histidine solution. Also a careful distinction must be made between the orange red color that histidine gives with Hunter's diazo reagent for ergothioneine and the bright purple red color given by ergothioneine. In addition, even dilute test solutions of ergothioneine smell of trimethylamine on standing with the strong sodium hydroxide used in the test.

The filtrate from the silver lactate precipitate was examined. A large quantity of creatinine was removed from the solution as the monopicrate, care being taken to keep the volume and temperature low to limit the solubility of creatinine monopicrate. The filtrate was freed of excess picric acid by the addition of hydrochloric acid and extraction of the picric acid with benzene. The solution was then clarified with bone charcoal and reduced to a small volume. The addition of absolute alcohol brought down a white precipitate which proved to be histidine. No ergothioneine was detected.

CONCLUSIONS AND SUMMARY

1. An investigation was made of the basic lead precipitate from urine.
2. A crystalline potassium salt introduced from the reagents has been described.
3. The strong acidity of the freed lead salts, thought to be due to proteic acids, is due largely to hydrochloric acid arising from the chloride in urine.
4. The unstable material from lead, mercury, and silver precipitation methods yielded no new substances.
5. Most of the organic sulphur compounds are to be found in the supernatant from lead precipitation at pH 9.0.
6. A fractionation of the supernatant after a preliminary lead precipitation yielded no new sulphur compounds.

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